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Closing the border on a new frontier: the problem with salivary nerve growth factor

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In 2013, a study appeared in Psychosomatic Medicine showing that salivary levels of Nerve Growth Factor (sNGF) rapidly increase (to +45% of baseline levels) in response to an acute psychosocial stress task that involved discussing an unresolved conflict with a romantic partner (1). NGF, also referred to as mature NGF- β , is a neurotrophic factor that, amongst other functions, mediates neuronal growth and differentiation, although it may also drive cell death (2). The study presented a genuinely novel finding, which was emphasized by the authors' branding of sNGF as a "new frontier for stress research" that could "represent a resilience factor protecting individuals from adverse effects of stress" (1, 3). Secondary analyses of these seminal data were in line with these speculations, showing, for example, that greater sNGF reactivity to conflict was related to stronger appraisals of coping ability and agency, lower anticipatory stress appraisals, and to higher wellbeing (1, 3). There is one problem, which is substantial: saliva appears to contain little, if any, NGF. Therefore, on grounds that the findings are biologically implausible, we think that the validity of these data (and related data published in other journals) need to be reconsidered.

While our main concern pertains to the validity of measuring NGF- β in saliva (discussed further below) there are two other issues that we would like to comment on; namely, the interpretation of stress-induced changes in salivary NGF- β , and statistical/design issues of the aforementioned study (1). First, we noticed that speculations regarding the role of human salivary NGF- β as presented by Laurent *et al* (1), as well in the subsequent articles (3-6), seemed inconsistent with current knowledge in this area and were based, in part, on extrapolations from studies in mice. In these animals the submaxillary salivary gland produces high concentrations of NGF- β , thought to have important local and endocrine functions (7). However, there are no human data

to suggest a biological role for salivary NGF- β in any other part of the body, and there is also no evidence that NGF- β levels in human saliva correspond with the levels in other compartments (e.g., blood, brain). Likewise, speculations about the protective roles of salivary NGF- β did not seem to be well-supported. An example is the statement that “a neurotrophic response to acute stress – found at both central and peripheral levels – may have evolved as a resilience mechanism to protect the brain and other tissues against cortisol release” (1). The biological effects of NGF- β (e.g., cell growth *versus* cell death) are highly contextual, and depend on NGF- β concentration, differential activation of its receptors (Trka and p75NTR), and signals from other growth factors and pro- or anti-growth receptors (2).

Second, the aforementioned article (1) raises statistical and methodological concerns. For example, the observation that salivary NGF- β increases in the stress group (N=40) but not in the control group (N=20) would be more convincing if a significant time by condition interaction was demonstrated (i.e., to indicate that the two groups differ). Such control-group comparisons were, however, not presented in the Psychosomatic Medicine article or in subsequent stress studies (1, 3-6). Further, the possible confounding role of saliva flow deserves more careful consideration in studies examining stress-related salivary responses, because reductions in saliva flow rate (e.g., as a result of stress) may cause artefactual increases in the concentration of salivary constituents (8, 9).

The primary issue, however, is the biological plausibility of salivary NGF- β itself. Doubts over the biological relevance of salivary NGF- β in humans have been long standing (10), in part because the levels appear to be a 1000-fold lower than in mice (11, 12). However, even these low human values are now disputed. The stress study in

Psychosomatic Medicine (1), and all subsequent studies (3-6), used an immunoassay produced by Promega (NGF E_{max} immunoassay system, Promega Madison, WI, USA), whereby the test method for use with saliva was developed by Salimetrics (3). As explained by Boreli et al. (13), only studies that have used the Promega immunoassay reported detectable levels of NGF- β in saliva. This company recently withdrew their assay from the market, and since then research groups have been unable to detect salivary NGF- β using other commercially available immunoassays or by using other techniques, such as western blotting (13, 14; and references therein). Only the precursor to mature NGF- β – pro-nerve growth factor (pro-NGF) – has been found in detectable amounts in saliva (14). Thus, the validation of the NGF E_{max} assay for use with saliva (1, 3) would have benefited from additional testing, such as cross-checking with other available immunoassays or checks using western blotting. Admittedly, the fact that saliva does not contain mature NGF- β , despite the presence of its precursor, is somewhat surprising, as we and others have found that the enzymes required for the splicing of pro-NGF into mature NGF- β (e.g., MMP-2) are abundant in saliva (15).

If NGF- β is not detectable in saliva, what then, might explain the anomalous results by Laurent and colleagues? The answer remains elusive. Some authors have speculated that the antibody in the Promega immunoassay may have been cross-reacting with pro-NGF (13, 14). If this were the case, then the results of Laurent et al (1) require a fundamental re-interpretation, because the biological effects of pro-NGF are different to its mature counterpart. For example, some actions of pro-NGF are the opposite of mature NGF- β , and elevated expression of pro-NGF has been implicated in several (neuro-)pathologies (16). However, many other possibilities remain, as saliva is notoriously 'sticky' and prone to generating non-specific signals in immunoassays. In

fact, Laurent and coworkers themselves may have spotted that something was amiss. In a paper that involved post-hoc analyses of their work published in *Psychosomatic Medicine* (3), the authors state “Our subsequent work with this assay reveals an issue overlooked by prior research – the antibody used in this assay system cross-links with sIgA.” Surprisingly, the authors dismissed this as a problem, arguing that the sIgA response to acute stress is different from the salivary cortisol response to stress, the latter of which the authors claimed salivary NGF mirrors (3).

Whatever the case, it remains that in humans there should be little to no detectable NGF- β in saliva. It is therefore unclear which biological marker or process, if anything, is being measured with the salivary NGF assay used. Consequently, reported correlates of basal salivary NGF- β , such as body mass index (5) and the levels of dehydroepiandrosterone sulfate (DHEA-S) (6) are void. Similarly, conclusions such as salivary NGF- β is reactive to stress (1), and that these reactions are related to factors like negative emotion (1), appraisals of stress and coping ability (3), gender (5), prior exposure to trauma (4), and genetic polymorphisms (4) need to be revisited. The number of investigations in the bio-psycho-social literature reporting correlates and predictors of salivary NGF- β continues to accumulate (1, 3-6, 17), and this letter is intended to stop this growth. Issues with the biological interpretation of saliva-based measures are not unique in the literature. An example is salivary alpha-amylase, a purported saliva measure of sympathetic activity; a claim which from the start was controversial, but nevertheless became presumed factual knowledge and difficult to correct or presented with important nuances (8, 9).

In closing, this letter is written in the spirit of collegial discourse and concerns the

scientific merit of the study published in this journal and the interpretation of salivary NGF, and not the quality of this team of investigators.

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